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**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US97/17162 <b>(22) International Filing Date:</b> 24 September 1997 (24.09.97)  <b>(30) Priority Data:</b> 60/027,657 4 October 1996 (04.10.96) US 08/771,850 23 December 1996 (23.12.96) US  <b>(71) Applicant:</b> LOMA LINDA UNIVERSITY [US/US]; Loma Linda, CA 92350 (US).  <b>(72) Inventors:</b> SZALAY, Aidar, A.; 7327 Fainwood, Highland, CA 92346 (US). WANG, Gefu; 1460 West Orange Avenue #56, Redlands, CA 92373 (US). WANG, Yubao; 24929 Academy Street, Loma Linda, CA 92354 (US).  <b>(74) Agents:</b> FARAH, David, A. et al.; Sheldon & Mak, 9th floor, 225 South Lake Avenue, Pasadena, CA 91101 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> RENILLA LUCIFERASE AND GREEN FLUORESCENT PROTEIN FUSION GENES  <b>(57) Abstract</b> <p>A fusion gene is provided comprising the cDNA of <i>Renilla</i> luciferase and the cDNA of the "humanized" <i>Aequorea</i> green fluorescent protein. The fusion gene was used to produce a novel protein, the "Renilla-GFP fusion protein", which displayed both the luciferase activity of <i>Renilla</i> luciferase, and the green fluorescence of GFP. The Renilla-GFP fusion gene is useful as a double marker for monitoring gene expression quantitatively in UV light and by enzyme activity.</p>		

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## RENILLA LUCIFERASE AND GREEN FLUORESCENT PROTEIN FUSION GENES CROSS-REFERENCE TO RELATED APPLICATIONS

The present Application is a International Application corresponding to United States Patent Application 08/771,850, filed December 23, 1996, entitled "The Construction and Expression of Renilla Luciferase and Green Fluorescent Protein Fusion Genes"; and is a Continuation-in-Part of United States Provisional Patent Application 60/027,657, filed October 4, 1996, entitled "The Construction and Expression of Renilla Luciferase and Green Fluorescent Fusion Genes in *E. coli* and Mammalian Cells," the contents of which are incorporated herein by reference in their entirety.

### BACKGROUND

Green Fluorescent Protein (GFP) is a light emitting protein purified from the jellyfish *Aequorea victoria*. GFP can emit green light by accepting energy transfer from sources that include exogenous blue light and *Renilla* luciferase catalyzed reactions. The gene for GFP was cloned and its cDNA is a powerful reporter gene in a variety of living systems, including bacteria, fungi, and mammalian tissues. The UV light stimulated GFP fluorescence does not require cofactors and the gene product alone can be sufficient to allow detection of living cells under the light microscope.

By modifying the wild type GFP protein, red-shifted GFP variants with bright emission have also been produced. These variants include EGFP, GFPS65T and RSGF. Recently, GFP was expressed in a human cell line and *in vivo*. C. Kaether, H.H. Gerdes. Visualization of protein transport along the secretory pathway using green fluorescent protein. FEBS-Lett. 1995; 369:267-71. "Humanized" GFP was synthesized with nucleotide changes that did not change the amino acid sequences with one exception.

*Renilla* luciferase is an enzyme purified from *Renilla reniformis*. The enzyme catalyzes the oxidative decarboxylation of coelenterazine in the presence of oxygen to produce blue light with an emission wavelength maximum of 478 nm. In *Renilla reniformis* cells, however, this reaction is shifted toward the green with a wavelength maximum of 510 nm due to an energy transfer to a Green Fluorescent Protein.

The gene for *Renilla* luciferase (*ruc*) was cloned and its cDNA was shown to be useful as a reporter gene in various living systems. D.C. Prasher, V.K. Eckenrode, W.W. Ward, F.G. Prendergast, M.J. Cormier. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 1992; 111:229-33. By providing appropriate promoters to the

cDNA as gene cassettes, the gene was expressed in bacteria, transformed plant cells, and mammalian cells. The high efficiency of *Renilla* luciferase is a useful trait as a marker enzyme for gene expression studies.

Given the properties of GFP and *Renilla* luciferase, it would be useful to have a single protein combining the functions of both *Renilla* luciferase enzymes and GFP to monitor gene expression quantitatively by UV light excitation or qualitatively by enzyme activity measurements.

### SUMMARY

According to one embodiment of the present invention, there are provided fusion gene constructs comprising the cDNA of *Renilla* luciferase and the cDNA of the "humanized" *Aequorea* green fluorescent protein. The fusion gene constructs were used to transform both prokaryotic and eukaryotic cells. One construct was expressed as a polypeptide having a molecular weight of about 65 kDa. This polypeptide, the "Renilla-GFP fusion protein," was bifunctional, displaying both the luciferase activity of *Renilla* luciferase and the green fluorescence of GFP. The Renilla-GFP fusion gene is useful as a double marker for monitoring gene expression in living cells and quantitatively by enzymatic activity.

The invention includes a protein comprising a polypeptide having both luciferase and GFP activities, or biologically active variants of a polypeptide having both luciferase and GFP, or a protein recognized by a monoclonal antibody having affinity to the polypeptide having both luciferase and GFP activities. The polypeptide can be made by recombinant DNA methods.

The invention further includes a high affinity monoclonal antibody that immunoreacts with the polypeptide. The antibody can have an Fc portion selected from the group consisting of the IgM class, the IgG class and the IgA class. The invention also includes a high affinity monoclonal antibody that immunoreacts with a polypeptide having both luciferase and GFP activities.

The invention further includes a polynucleotide sequence coding for a polypeptide having both luciferase and GFP activities, or its complementary strands, and a polynucleotide sequence that hybridizes to such a sequence and that codes on expression for a polypeptide having both luciferase and GFP activities, or its complementary strands.

The invention further includes a purified and isolated DNA molecule comprising a polynucleotide coding for a polypeptide having both luciferase and GFP

activities, or its complementary strands. The polynucleotide can comprise the sequence as set forth in SEQ ID NO:1.

The invention further includes a vector containing a DNA molecule coding for a polypeptide having both luciferase and GFP activities. The polynucleotide can comprise the sequence as set forth in SEQ ID NO:1. The vector can be used to stably transform or transiently transfect a host cell.

The invention further includes a method of making a polypeptide having both luciferase and GFP activities. The method comprises the steps of, first, culturing a microorganism transformed with a polynucleotide vector containing a gene cassette coding for a polypeptide having both luciferase and GFP activities. Next, the polypeptide having both luciferase and GFP activities is recovered.

The invention further includes a method of quantifying promoter activations and GFP fluorescence based on luciferase activity measurements. The method comprises the step of providing the polypeptide according to the present invention.

The invention further includes a method of making a monoclonal antibody that immunoreacts with a polypeptide having both luciferase and GFP activities. The method comprises the steps of, first, administering to a host a polypeptide having both luciferase and GFP activities in an amount sufficient to induce the production of antibodies to the polypeptide from the host's antibody-producing cells. Next, the antibody-producing cells are recovered from the host. Then, cell hybrids are formed by fusing the antibody-producing cell to cells capable of substantially unlimited reproduction. Then, the hybrids are cultured. Next, the monoclonal antibodies are collected as a product of the hybrids.

The invention further includes a method of monitoring gene expression quantitatively and qualitatively in a cell using a gene fusion construct coding for a polypeptide having both luciferase and GFP activities. The method comprises the steps of, first, providing a gene fusion construct coding for a polypeptide having both Renilla luciferase and GFP activity. Next, the gene fusion construct is introduced into the cell. Then, the cell containing the gene fusion construct is maintained in a manner allowing the cell to express the polypeptide. Then, the cell is measured for luciferase and fluorescent activity. The construct can include a polynucleotide sequence as set forth in SEQ ID NO:1.

The invention further includes a method of monitoring gene expression quantitatively and qualitatively in a cell using a gene fusion construct coding for a polypeptide

having both luciferase and GFP activities. The method comprises the steps of, first, providing a gene fusion construct coding for a polypeptide having both luciferase and GFP activities. Next, the gene fusion construct is introduced into the cell. Then, the cell containing the gene fusion construct is maintained in a manner allowing the cell to express the polypeptide. Next, the cell is measured for luciferase and fluorescent activity.

## FIGURES

These and other features, aspects and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying figures where:

10 Figure 1 is a schematic diagram showing the construction of a *Renilla* luciferase and "humanized" GFP fusion gene cassette according to the present invention for gene expression in *E. coli* where "RG," top, is the fusion gene cassette with the *Renilla* luciferase coding sequence (ruc) at the 5' terminus, and "GR," bottom, is the fusion gene cassette with the GFP coding sequence (gfp<sub>h</sub>) at the 5' terminus;

15 Figure 2 is a schematic diagram showing the construction of *Renilla* luciferase and "humanized" GFP fusion gene cassette according to the present invention for gene expression in mammalian cells where "RG," top, is the fusion gene cassette with the *Renilla* luciferase coding sequence (ruc) at the 5' terminus, and "GR," bottom, is the fusion gene cassette with the GFP coding sequence (gfp<sub>h</sub>) at the 5' terminus;

20 Figure 3 is a map of the plasmids used for cloning and expression of the RG gene construct in *E. coli* (top) and the GR gene construct in *E. coli* (bottom);

Figure 4 is a map of the plasmids used for cloning and expression of the RG gene construct in mammalian systems (top) and the GR gene construct in mammalian systems (bottom);

25 Figure 5 are photomicrographs of cells transformed by the fusion genes using fluorescence microscopy and fluorescence imaging to show GFP activity;

Figure 6 are bar graphs of luciferase activity of the fusion gene constructs in *E. coli* (top) and mammalian cells (bottom);

30 Figure 7 is a spectroscopic measurement of *Renilla* luciferase activity and GFP activity in *E. coli*;

Figure 8 is a Western blot showing the detection of fusion gene expression in *E. coli* using anti-*Renilla* luciferase antibody;



Figure 9 are photomicrographs of mouse embryonic stem cells using fluorescence image analysis demonstrating the expression of the RG fusion gene; and

Figure 10 are photomicrographs of mouse embryos using fluorescence image analysis demonstrating the expression of the RG fusion gene.

## DESCRIPTION

According to one embodiment of the present invention, there is provided a fusion gene comprising the cDNA of *Renilla* luciferase and the cDNA of the "humanized" *Aequorea* green fluorescent protein. According to another embodiment of the present invention, there is provided a single polypeptide that exhibits both *Renilla* luciferase and GFP activities. This bifunctional polypeptide can facilitate the identification of transformed cells at the single cell level, in cell cultures, transformed tissues and organs based on fluorescence of the polypeptide. At the same time, the polypeptide can also be used to quantify promoter activations and GFP fluorescence based on luciferase activity measurements.

The cDNA of *Renilla reniformis* luciferase (ruc) has been cloned and used successfully as a marker gene in a variety of transgenic species. See, for example, Lorenz, W.W., McCann, R.O., Longiaru, M. and Cormier, M.J. Isolation and expression of a cDNA encoding *Renilla reniformis* luciferase. Proc. Natl. Acad. Sci. USA 1991; 88:4438-4442; Mayerhofer, R., Langridge, W.H.R., Cormier, M.J., and Szalay, A.A. Expression of recombinant *Renilla* luciferase in transgenic plants results in high levels of light emission. The Plant Journal 1995; 7:1031-1038; and Lorenz, W.W., Cormier, M.J., O'Kane, D.J., Hua, D., Escher, A. A. Szalay, A.A. Expression of the *Renilla reniformis* luciferase gene in mammalian cells. J. Biolumin. Chemilumin. 1995; 11:31-37, incorporated herein by reference in their entirety. Similarly, the transfer and expression of Green-Fluorescent-Protein (GFP) cDNA from *Aequorea victoria* resulted in high levels of GFP in transformed cells that allowed convenient visualization of individual cells under the microscope. See, for example, Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. Green fluorescent protein as a marker for gene expression. Science 1994; 263:802-805, incorporated herein by reference in its entirety.

The present invention involves the production of fusion genes from the cDNA of *Renilla* (ruc) and the cDNA of the "humanized" *Aequorea* GFP (gfp<sub>h</sub>). A description of "humanized" *Aequorea* GFP (gfp<sub>h</sub>) can be found, for example, in Zolotukhin, S., Potter, M., and Huaswirth, W.W., Guy, J., and Muzyczka, N. A "humanized" green fluorescent protein

cDNA adapted for high-level expression in mammalian cells. J. Virology 1996: 70:4646-4654, incorporated herein by reference in its entirety.

The first fusion gene, designated the "RG fusion gene," SEQ ID NO:1 and shown at the top of Figures 1 and 2, contains the *Renilla* cDNA linked at the modified 3' end to a fifteen polynucleotide linker sequence encoding five amino acids, Ala-Ala-Ala-Ala-Thr, residues 312-316 of SEQ ID NO:1, followed by the 5' end of the intact GFP cDNA. The second fusion gene, designated the "GR fusion gene," SEQ ID NO:2 and shown at the bottom of Figures 1 and 2, contains the cDNA of GFP linked to a twenty-seven polynucleotide linker sequence encoding nine amino acids, Gly-Try-Gln-Ile-Glu-Phe-Ser-Leu-Lys, residues 239-247 of SEQ ID NO:2, followed by the 5' end of *Renilla* cDNA. Both genes were placed into prokaryotic pGEM-5zf(+) and eukaryotic pCEP4 expression vectors, and transformed into *E. coli*, and various mammalian cell lines, and microinjected into mouse embryos. PT<sub>7</sub> was the bacterial T7 promoter used for gene expression. P<sub>cmv</sub> was the CMV promoter used for gene expression in mouse fibroblast cells, embryonic stem cells and mouse embryos.

Unexpectedly, only cells transformed with the RG fusion gene gave strong fluorescence while the cells containing the GR fusion gene exhibited minimal response to UV light under the microscope. In contrast, luciferase measurements in homogenates of cells transformed with RG gene cassettes or with GR gene cassettes were indistinguishable from each other in both bacterial and mammalian cells. Further, spectrofluorimeter data indicated that there was energy transfer between *Renilla* luciferase and GFP in the RG fusion gene containing cells but did not indicate such energy transfer in cells containing the GR fusion gene. The protein expressed in the RG fusion gene containing cells was analyzed and found to be a 65 kDa polypeptide. A detailed description of the construction and expression of the fusion genes, and analyses of their protein products is given below.

#### Production of the Fusion Gene Constructs:

The vectors used for cloning and expression of the gene constructs in *E. coli* and mammalian systems were pGEM-5zf(+) (Promega) and pCEP4, respectively. Figure 3 is a map of the plasmids used for cloning and expression of the RG gene construct in *E. coli*, pGEM-5zf(+)-RG (top) and the map of the plasmids used for cloning and expression of the GR gene construct in *E. coli*, pGEM-5zf(+)-GR (bottom). Both were under the transcriptional control of T7 promoter. The *E. coli* strains which were transformed were DLT101 and DH5 $\alpha$ .

Similarly, Figure 4 is a map of the plasmids used for cloning and expression of the RG gene construct in mammalian systems, pCEP4-RG (top), and a map of the plasmids used for cloning and expression of the GR gene construct in mammalian systems, pCEP4-GR (bottom). Both were under the transcriptional control of CMV promoter. The mammalian cell line that was transformed was LM-TK embryonic stem cells and embryos.

Five primers were designed for cloning the RG and GR gene constructs. Single underlines indicate Shine-Dalgarno sequences. Double underlines indicate the restriction sites. The start codons are in bold. Sequences in bold italics indicate the removal of stop codons from both *luc* and *gfp* genes.

- 10 Primer 1, SEQ ID NO:3: RUC5: 5'CTGCAG (PstI)  
AGGAGGAATTCAGCTTAAAGATG3'
- Primer 2, SEQ ID NO:4: RUC3: 5'GCGGCCGC (Not I) **T**TG TTCATTTTGTGAGAAC3'
- Primer 3, SEQ ID NO:5: GFP5: 5'GGGGTACC (KpnI)  
CCATGACCAAGGGGCGAGGAACT3'
- 15 Primer 4, SEQ ID NO:6: GFP3: 5'GGGGTACC (KpnI)  
CCTTGTACAGCTCGTCCATGCCA3'
- Primer 5, SEQ ID NO:7: GFP5a 5' CCCGGG (SmaI)  
AGGAGGTACCCCATGAGCAAG3'

The *Renilla* luciferase-GFP fusion gene (RG gene cassette) and the GFP-

- 20 *Renilla* luciferase fusion gene (GR gene cassette) were constructed by removing the stop codons, and by adding restriction sites and Shine-Dalgarno sequences to the 5' end of the cDNAs using PCR according to techniques known to those with skill in the art. The PCR products were cloned using the pGEM-T system (Promega Corporation, Madison, WI).

Primers were designed so that the downstream cDNA is in frame with the upstream cDNA.

- 25 The linker sequences are shown in Figures 1 and 2 and described above. After cloning, the RG and GR gene cassettes were under the transcriptional control of T7 in pGEM-5zf(+). vector and CMV in pCEP4 vector, which were used for expression in *E. coli* and mammalian cells, respectively.

#### Determination of activity of fusion genes and their corresponding protein products:

- 30 GFP activity *in vivo* was visualized as follows. *E. coli* strain DH5 $\alpha$  was transformed with the plasmids pGEM-5zf(+)-RG and pGEM-5zf(+)-GR. Positive colonies were identified and cultured in LB medium with 100  $\mu$ g/ml of ampicillin selection, according

to techniques known to those with skill in the art. Twelve hours later, one drop of *E. coli* culture was put on a slide and visualized by fluorescent microscopy at 1000 x magnification.

LM-TK cells were transfected with plasmids pCEP4-RG and pCEP4-GR using calcium phosphate methods known to those with skill in the art. The culture dishes were monitored using an inverted fluorescent microscope 12 hours after the transfection.

Luciferase activity was assayed as follows. An aliquot of transformed *E. coli* was used for a luciferase assay in a Turner TD 20e luminometer (Turner Designs, Sunnyvale, CA), both before and after IPTG induction. The results were recorded as relative light units. Mammalian cells harvested 36 hrs after transfection were measured for luciferase activity.

Corrected emission spectra were detected spectrofluorimetrically using a SPEX fluorolog spectrofluorimeter operated in the ratio mode. Fluorescence emission was excited at 390 nm. Bioluminescence emission was recorded with the excitation beam blocked following the addition of 0.1  $\mu$ g of coelenterazine in acidified methanol. Five spectra were averaged for each sample over a wavelength range from 400 to 600 nm.

The fusion proteins were isolated and immunoactivity detected as follows. 1 ml of *E. coli* ( $OD_{600} = 1.0$ ) was harvested. 400  $\mu$ l of cell suspension buffer (0.1M NaCl, 0.01 M Tris-HCl pH 7.6, 0.001 M EDTA, 100  $\mu$ g/ml PMSF) and 100  $\mu$ l of loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) were added. The samples were boiled for 4 min and loaded to a 7.5%-20% gradient SDS-polyacrylamide gel.

Polyclonal anti-*Renilla* luciferase was used as the primary antibody for detection and goat peroxidase anti-IgG (anti-rabbit) as the secondary antibody.

Referring now to Figure 5, there are shown photomicrographs of GFP activity in transformed *E. coli* cells (5A, left side) and LM-TK mouse fibroblast cells (5B, right side) by fluorescence microscopy and fluorescence imaging. As can be seen, individual *E. coli* cells and mammalian cells transformed with the RG fusion gene construct exhibited strong green fluorescence under oil immersion.

Referring now to Figure 6, there are shown bar graphs of luciferase activity of the gene constructs in *E. coli* (top) and mammalian cells (bottom). The white bars indicate activity before promoter induction. The black bars indicate activity after promoter induction.

As can be seen, cells transformed with the RG fusion gene construct have significant luciferase activity, which is reduced 3-fold in the cells transformed with the GR fusion gene construct.

Referring now to Figure 7, there is shown a spectroscopic measurement of *Renilla* luciferase activity and GFP activity in *E. coli* transformed with various gene constructs. As can be seen, cells containing *Renilla* luciferase gene (short dashes) show only one emission peak at approximately 478 nm. Cells containing the GR gene fusion construct (light solid) also show one emission peak at approximately 478 nm, indicating *Renilla* luciferase activity only. By contrast, cells containing the RG gene fusion construct (heavy solid) show an emission peak at approximately 510 nm with excitation at 390 nm. Cells containing the RG gene fusion construct with the addition of coelenterazine (long dashes) show emission peaks at both approximately 478 nm and 510 nm indicating that the energy transfer between *Renilla* luciferase and GFP occurred in these cells. The lack of GFP activity in GR gene cassette transformed cell lines could be due to incorrect folding, due to the requirement for a free GFP C-terminus, or due to interference of the linker polypeptide with GFP activity in the fusion protein, among other possible explanations.

Referring now to Figure 8, there is shown a western blot used to detect fusion gene expression in *E. coli* using anti-*Renilla* luciferase antibody. Reading from left to right, the "C" lane shows the total protein extracted from non-transformed *E. coli* cells. The "R" lane shows the total protein extracted from *E. coli* cells transformed with the *ruc* gene alone. The "G" lane shows the total protein extracted from *E. coli* cells transformed with the *gfp* gene alone. The "RG" lane shows the total protein extracted from *E. coli* cells transformed with the RG fusion gene cassette. The "GR" lane shows the total protein extracted from *E. coli* cells transformed with the GR fusion gene cassette.

As can be seen, protein extracted from *E. coli* cells transformed with the *ruc* gene alone produced a band with a molecular weight of about 34 kDa. Protein extracted from *E. coli* cells transformed with the RG fusion gene cassette produced a band with a molecular weight of about 65 kDa. Protein extracted from *E. coli* cells transformed with the GR fusion gene cassette produced a band with a molecular weight of about 34 kDa. These data imply that cells transformed with the GR fusion gene cassette produced luciferase but did not produce fusion protein. Such a lack of fusion protein production by cells transformed with the GR fusion cassette would explain the lack of green fluorescent activity in these cells.

Referring now to Figure 9, there are shown photomicrographs using fluorescence image analysis demonstrating the expression of the RG fusion gene in mouse

embryonic stem cells transformed by electroporation procedures. Transformed colonies were selected based on GFP activity under fluorescence microscopy.

Referring now to Figure 10, there are shown photomicrographs using fluorescence image analysis demonstrating the expression of the RG fusion genes in mouse embryos. The embryos were injected with the linearized RG plasmid, and *in vitro* cultured. The expression of GFP activity was monitored daily by fluorescent microscope and recorded by an imaging collection system.

Based on this data, we conclude that the RG fusion construct disclosed herein can be expressed in both prokaryotic and eukaryotic cells to produce a bifunctional polypeptide that exhibits both *Renilla* luciferase and GFP activity. This bifunctional polypeptide is a useful tool for identification of transformed cells at the single cell level based on fluorescence. It allows the simultaneous quantification of promoter activation in transformed tissues and transgenic organisms by measuring luciferase activity. The dual function of this protein allows the monitoring of bacterial cells in their living hosts and the differentiation of cells in the developing embryo and throughout the entire animal.

Although the present invention has been discussed in considerable detail with reference to certain preferred embodiments, other embodiments are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of preferred embodiments contained herein.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Szalay, Aladar A.  
Wang, Gefu  
Wang, Yubao
- (ii) TITLE OF INVENTION: THE CONSTRUCTION AND EXPRESSION OF RENILLA LUCIFERASE AND GREEN-FLUORESCENT PROTEIN-FUSION GENES
- (iii) NUMBER OF SEQUENCES: 7
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(A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 Mb storage  
(B) COMPUTER: IBM compatible  
(C) OPERATING SYSTEM: Windows 95  
(D) SOFTWARE: WordPerfect for Windows version 6.1
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER: to be assigned  
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- (vii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Farah, David A.  
(B) REGISTRATION NUMBER: 38,134  
(C) REFERENCE/DOCKET NUMBER: 11385-1PCT
- (ix) TELECOMMUNICATION INFORMATION:  
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(B) TELEFAX: 626/795-6321

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1665 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

ATG ACT TCG AAA GTT TAT GAT CCA GAA CAA AGG AAA CGG ATG ATA ACT      48
Met Thr Ser Lys Val Tyr Asp Pro Gln Arg Lys Arg Met Ile Thr
1          5          10
GGT CCG CAG TGG TGG GCC AGA TGT AAA CAA ATG AAT GTT CTT GAT TCA      96
Gly Pro Gln Trp Trp Ala Arg Cys Lys Gln Met Asn Val Leu Asp Ser
20          25          30
TTT ATT AAT TAT TAT GAT TCA GAA AAA CAT GCA GAA AAT GCT GTT ATT      144
Phe Ile Asn Tyr Tyr Asp Ser Glu Lys His Ala Glu Asn Ala Val Ile
35          40          45
TTT TTA CAT GGT AAC GCG GCC TCT TCT TAT TTA TGG CGA CAT GTT GTG      192
Phe Leu His Gly Asn Ala Ala Ser Ser Tyr Leu Trp Arg His Val Val
50          55          60
CCA CAT ATT GAG CCA GTA GCG CGG TGT ATT ATA CCA GAT CTT ATT GGT      240
Pro His Ile Glu Pro Val Ala Arg Cys Ile Ile Pro Asp Leu Ile Gly
65          70          75          80
ATG GGC AAA TCA GGC AAA TCT GGT AAT GGT TCT TAT AGG TTA CTT GAT      288
Met Gly Lys Ser Gly Lys Ser Gly Asn Gly Ser Tyr Arg Leu Leu Asp
85          90          95

```

CAT TAC AAA TAT CTT ACT GCA TGG TTT GAA CTT CTT AAT TTA CCA AAG	336
His Tyr Lys Tyr Leu Thr Ala Trp Phe Glu Leu Leu Asn Leu Pro Lys	
100 105 110	
AAG ATC AAT TTT GTC GGC CAT GAT TGG GGT GCT TGT TTG GCA TTT CAT	384
Lys Ile Ile Phe Val Gly His Asp Trp Gly Ala Cys Leu Ala Phe His	
115 120 125	
TAT AGC TAT GAG CAT CAA GAT AAG ATC AAA GCA ATA GTT CAC GCT GAA	432
Tyr Ser Tyr Glu His Gln Asp Lys Ile Lys Ala Ile Val His Ala Glu	
130 135 140	
AGT GTA GTA GAT GTG ATT GAA TCA TGG GAT GAA TGG CCT GAT ATT GAA	480
Ser Val Val Asp Val Ile Glu Ser Trp Asp Glu Trp Pro Asp Ile Glu	
145 150 155 160	
GAA GAT ATT GCG TTG ATC AAA TCT GAA GAA GGA GAA AAA ATG GTT TTG	528
Glu Asp Ile Ala Leu Ile Lys Ser Glu Glu Gly Glu Lys Met Val Leu	
165 170 175	
GAG AAT AAC TTC TTC GTG GAA ACC ATG TTG CCA TCA AAA ATC ATG AGA	576
Glu Asn Asn Phe Phe Val Glu Thr Met Leu Pro Ser Lys Ile Met Arg	
180 185 190	
AAG TTA GAA CCA GAA GAA TTT GCA GCA TAT CTT GAA CCA TTC AAA GAG	624
Lys Leu Glu Pro Glu Glu Phe Ala Ala Tyr Leu Glu Pro Phe Lys Glu	
195 200 205	
AAA GGT GAA GTT CGT CGT CCA ACA TTA TCA TGG CCT CGT GAA ATC CCG	672
Lys Gly Glu Val Arg Arg Pro Thr Leu Ser Trp Pro Arg Glu Ile Pro	
210 215 220	
TTA GTA AAA GGT GGT AAA CCT GAC GTT GTA CAA ATT GTT AGG AAT TAT	720
Leu Val Lys Gly Gly Lys Pro Asp Val Val Gln Ile Val Arg Asn Tyr	
225 230 235 240	
AAT GCT TAT CTA CGT GCA AGT GAT GAT TTA CCA AAA ATG TTT ATT GAA	768
Asn Ala Tyr Leu Arg Ala Ser Asp Asp Leu Pro Lys Met Phe Ile Glu	
245 250 255	
TGG GAT CCA GGA TTC TTA TCC AAT GCT ATT GTT GAA GGC GCG AAG AAG	816
Ser Asp Phe Gly Phe Phe Ser Asn Ala Ile Val Glu Gly Ala Lys Lys	
260 265 270	
TTT CCT AAT ACT GAA TTT GTC AAA GTA AAA GGT CTT CAT TTT TCG CAA	864
Phe Pro Asn Thr Glu Phe Val Lys Val Lys Gly Leu His Phe Ser Gln	
275 280 285	
GAA GAT GCA CCT GAT GAA ATG GGA AAA TAT ATC AAA TCG TGC GTT GAG	912
Glu Asp Ala Pro Asp Glu Met Gly Lys Tyr Ile Lys Ser Phe Val Glu	
290 295 300 305	
CGA GTT CTC AAA AAT GAA CAA GCG GCC GCC GCC ACC ATG AGC AAG GGC	960
Arg Val Leu Lys Asn Glu Gln Ala Ala Ala Thr Met Ser Lys Gly	
305 310 315 320	
GAG GAA CTG TTC ACT GGC GTG GTC CCA ATT CTC GTG GAA CTG GAT GGC	1008
Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val Glu Leu Asp Gly	
325 330 335	
GAT GTG AAT GGG CAC AAA TTT TCT GTC AGC GGA GAG GGT GAA GGT GAT	1056
Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu Gly Glu Gly Asp	
340 345 350	



GCC ACA TAC GGA AAG CTC ACC CTG AAA TTC ATC TGC ACC ACT GGA AAG 1104  
Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys Thr Thr Gly Lys 355 360 365

CTC CCT GTG CCA TGG CCA ACA CTG GTC ACT ACC TTC ACC TAT GGC GTG 1152  
Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe Thr Tyr Gly Val 370 375 380

CAG TGC TTT TCC AGA TAC CCA GAC CAT ATG AAG CAG CAT GAC TTT TTC 1200  
Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln His Asp Phe Phe 385 390 395 400

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Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe 405 410 415

AAA GAT GAC GGG AAC TAC AAG ACC CGC GCT GAA GTC AAG TTC GAA GGT 1296  
Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly 420 425 430

GAC ACC CTG GTG AAT AGA ATC GAG CTG AAG GGC ATT GAC TTT AAG GAG 1344  
Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu 435 440 445

GAT GGA AAC ATT CTG GGC CAC AAG CTG GAA TAC AAC TAT AAC TCC CAC 1392  
Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His 450 455 460

AAT GTG TAC ATC ATG GGC GAC AAG CAA AAG AAT GGC ATC AAG GTC AAC 1440  
Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn 465 470 475 480

TTC AAG ATC AGA CAC AAC ATT GAG GAT GGA TCC GTG CAG CTG GCC GAC 1488  
Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp 485 490 495

CAT TAT CAA CAG AAC ACT CCA ATC GGC GAC GGC CCT AGT GTC CTC CTC CCA 1536  
His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu Pro 500 505 510

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Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp Pro Asn 515 520 525

GAA AAG AGA GAC CAC ATG GTC CTG CTG GAG TTT GTG ACC GCT GCT GGG 1632  
Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr Ala Ala Gly 530 535 540

ATC ACA CAT GGC ATG GAC GAG CTG TAC AAG TGA 1665  
Ile Thr His Gly Met Asp Glu Leu Tyr Lys 545 550

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1677 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATG AGC AAG GGC GAG GAA CTG TTC ACT GGC GTG GTC CCA ATT CTC GTG 48  
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val  
1 5 10 15

GAA CTG GAT GGC GAT GTG AAT GGG CAC AAA TTT TCT GTC AGC GGA GAG	96
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu	
20 25 30	
GGT GAA GGT GAT GCC ACA TAC GGA AAG CTC ACC CTG AAA TTC ATC TGC	144
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys	
35 40 45	
ACC ACT GGA AAG CTC CCT GTG CCA TGG CCA ACA CTG GTC ACT ACC TTC	192
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe	
50 55 60	
ACC TAT GGC GTG CAG TGC TTT TCC AGA TAC CCA GAC CAT ATG AAG CAG	240
Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln	
65 70 75 80	
CAT GAC TTT TTC AAG AGC GGC ATG CCC GAG GGC TAT GTG CAG GAG AGA	288
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg	
85 90 95	
ACC ATC TTT TTC AAA GAT GAC GGG AAC TAC AAG ACC CGC GCT GAA GTC	336
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val	
100 105 110	
AAG TTC GAA GGT GAC ACC CTG GTG AAT AGA ATC GAG CTG AAG GGC ATT	384
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile	
115 120 125	
GAC TTT AAG GAG GAT GGA AAC ATT CTC GGC CAC AAG CTG GAA TAC AAC	432
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn	
130 135 140	
TAT AAC TCC CAC AAT GTG TAC ATC ATG GCC GAC AAG CAA AAG AAT GGC	480
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly	
145 150 155 160	
ATC AAG GTC AAC TTC AAG ATC AGA CAC AAC ATT GAG GAT GGA TCC GTG	528
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val	
165 170 175	
CAG CTG GCC GAC CAT TAT CAA CAG AAC ACT CCA ATC GGC GAC GGC CCT	576
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro	
180 185 190	
GTG CTC CTC CCA GAC AAC CAT TAC CTG TCC ACC CAG TCT GCC CTG TCT	624
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser	
195 200 205	
AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTG CTG GAG TTT GTG	672
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val	
210 215 220	
ACC GCT GCT GGG ATC ACA CAT GGC ATG GAC GAG CTG TAC AAG GGC TAC	720
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys Gly Tyr	
225 230 235 240	
CAG ATC GAA TTC AGC TTA AAG ATG ACT TCG AAA GTT TAT GAT CCA GAA	768
Gln Ile Glu Phe Ser Leu Lys Met Thr Ser Lys Val Tyr Asp Pro Glu	
245 250 255	
CAA AGG AAA CGG ATG ATA ACT GGT CCG CAG TGG TGG GCC AGA TGT AAA	816
Gln Arg Lys Arg Met Ile Thr Gly Pro Gln Trp Trp Ala Arg Cys Lys	
260 265 270	
CAA ATG AAT GTT CTT GAT TCA TTT ATT AAT TAT TAT GAT TCA GAA AAA	864
Gln Met Asn Val Leu Asp Ser Phe Ile Asn Tyr Tyr Asp Ser Glu Lys	

275	280	285	
CAT GCA GAA AAT GCT GTT ATT TTT TTA CAT GGT AAC GCG GCC TCT TCT			912
His Ala Glu Asn Ala Val Ile Phe Leu His Gly Asn Ala Ala Ser Ser			
290	295	300	
TAT TTA TGG CGA CAT GTT GTG CCA CAT ATT GAG CCA GTA GCG CGG TGT			960
Tyr Leu Trp Arg His Val Val Pro His Ile Glu Pro Val Ala Arg Cys			
305	310	315	320
ATT ATA CCA GAT CTT ATT GGT ATG GGC AAA TCA GGC AAA TCT GGT AAT			1008
Ile Ile Pro Asp Leu Ile Gly Met Gly Lys Ser Gly Lys Ser Gly Asn			
325	330	335	
GGT TCT TAT AGG TTA CTT GAT CAT TAC AAA TAT CTT ACT GCA TGG TTT			1056
Gly Ser Tyr Arg Leu Leu Asp His Tyr Lys Tyr Leu Thr Ala Trp Phe			
340	345	350	
GAA CTT CTT AAT TTA CCA AAG AAG ATC ATT TTT GTC GGC CAT GAT TGG			1104
Glu Leu Leu Asn Leu Pro Lys Lys Ile Ile Phe Val Gly His Asp Trp			
355	360	365	
GGT GCT TGT TTG GCA TTT CAT TAT AGC TAT GAG CAT CAA GAT AAG ATC			1152
Gly Ala Cys Leu Ala Phe His Tyr Ser Tyr Glu His Gln Asp Lys Ile			
370	375	380	
AAA GCA ATA GTT CAC GCT GAA AGT GTA GTA GAT GTG ATT GAA TCA TGG			1200
Lys Ala Ile Val His Ala Glu Ser Val Val Asp Val Ile Glu Ser Trp			
385	390	395	400
GAT GAA TGG CCT GAT ATT GAA GAA GAT ATT GCG TTG ATC AAA TCT GAA			1248
Asp Glu Trp Pro Asp Ile Glu Glu Asp Ile Ala Leu Ile Lys Ser Glu			
405	410	415	
GAA GGA GAA AAA ATG GTT TTG GAG AAT AAC TTC TTC GTG GAA ACC ATG			1296
Glu Gly Glu Lys Met Val Leu Glu Asn Asn Phe Phe Val Glu Thr Met			
420	425	430	
TTG CCA TCA AAA ATC ATG AGA AAG TTA GAA CCA GAA GAA TTT GCA GCA			1344
Leu Pro Ser Lys Ile Met Arg Lys Leu Glu Pro Glu Glu Phe Ala Ala			
435	440	445	
TAT CTT GAA CCA TTC AAA GAG AAA GGT GAA GTT CGT CGT CCA ACA TTA			1392
Tyr Leu Glu Pro Phe Lys Glu Lys Gly Glu Val Arg Arg Pro Thr Leu			
450	455	460	
TCA TGG CCT CGT GAA ATC CCG TTA GTA AAA GGT GGT AAA CCT GAC GTT			1440
Ser Trp Pro Arg Glu Ile Pro Leu Val Lys Gly Gly Lys Pro Asp Val			
465	470	475	480
GTA CAA ATT GTT AGG AAT TAT AAT GCT TAT CTA CGT GCA AGT GAT GAT			1488
Val Gln Ile Val Arg Asn Tyr Asn Ala Tyr Leu Arg Ala Ser Asp Asp			
485	490	495	
TTA CCA AAA ATG TTT ATT GAA TCG GAT CCA GGA TTG TTT TCC AAT GCT			1536
Leu Pro Lys Met Phe Ile Glu Ser Asp Pro Gly Phe Phe Ser Asn Ala			
500	505	510	
ATT GTT GAA GGC GCC AAG AAG TTT CCT AAT ACT GAA TTT GTC AAA GTA			1584
Ile Val Glu Gly Ala Lys Lys Phe Pro Asn Thr Glu Phe Val Lys Val			
515	520	525	
AAA GGT CTT CAT TTT TCG CAA GAA GAT GCA CCT GAT GAA ATG GGA AAA			1632
Lys Gly Leu His Phe Ser Gln Glu Asp Ala Pro Asp Glu Met Gly Lys			
530	535	540	

TAT ATC AAA TCG TTC GTT GAG CGA GTT CTC AAA AAT GAA CAA TAA 1677  
Tyr Ile Lys Ser Phe Val Glu Arg Val Leu Lys Asn Glu Gln \*\*\*  
545 550 555

(3) INFORMATION FOR SEQ ID NO:3:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTGCAGAGGA GGAATTCAGC TTAAAGATG

29

(4) INFORMATION FOR SEQ ID NO:4:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGGCCGCTT GTTCATTTTT GAGAAC

26

(5) INFORMATION FOR SEQ ID NO:5:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGGTACCCG ATGAGCAAGG GCGAGGAAGT

30

(6) INFORMATION FOR SEQ ID NO:6:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGGTACCCG TTGTACAGCT CGTCCATGEC

31

(7) INFORMATION FOR SEQ ID NO:7:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCGGGAGGA GGTACCCCAT GAGCAAG

27

**WE CLAIM:**

1. A protein comprising a polypeptide having both luciferase and GFP activities or biologically active variants thereof.
2. A recombinant protein according to claim 1.
- 5 3. A protein according to claim 1, having an amino acid sequence as set forth in SEQ ID NO:1.
4. A high affinity monoclonal antibody which immunoreacts with the polypeptide of claim 1.
5. The antibody of claim 4 having an Fc portion selected from the group consisting of  
10 the IgM class, the IgG class and the IgA class.
6. A protein recognized by a monoclonal antibody having affinity to the polypeptide of claim 1.
7. The protein of claim 1 in purified and isolated form.
8. A DNA sequence coding for a protein according to claim 1, or its complementary  
15 strands.
9. A DNA sequence which hybridizes to a DNA sequence according to claim 8 and which codes on expression for a polypeptide having both luciferase and GFP activities, or its complementary strands.
10. A high affinity monoclonal antibody which immunoreacts with a polypeptide  
20 having both luciferase and GFP activities.
11. A purified and isolated DNA molecule comprising a polynucleotide coding for a polypeptide having both luciferase and GFP activities, or its complementary strands.
12. The DNA of claim 11, wherein the polynucleotide comprises the sequence as set forth in SEQ ID NO:1.
- 25 13. A vector containing a DNA molecule coding for a polypeptide having both luciferase and GFP activities.
14. The vector of claim 13, wherein the polynucleotide comprises the sequence as set forth in SEQ ID NO:1.
15. A prokaryotic or eukaryotic host cell stably transformed or transfected by the  
30 vector of claim 13.
16. A method of making a polypeptide having both luciferase and GFP activities, the method comprising the steps of:

- (a) culturing a microorganism transformed with a polynucleotide coding for a polypeptide having both luciferase and GFP activities; and
- (b) recovering the polypeptide having both luciferase and GFP activities.

17. A method of quantifying promoter activations and GFP fluorescence based on luciferase activity measurements, the method comprising the step of providing the polypeptide according to claim 1.

18. A method of making a monoclonal antibody which immunoreacts with a polypeptide having both luciferase and GFP activities, the method comprising the steps of:

- (a) administering to a host a polypeptide having both luciferase and GFP activities in an amount sufficient to induce the production of antibodies to the polypeptide;
- (b) recovering the antibody-producing cells from the host;
- (c) forming cell hybrids by fusing the antibody-producing cell to cells capable of substantially unlimited reproduction;
- (d) culturing the hybrids; and
- (e) collecting the monoclonal antibodies as a product of the hybrids.

19. A method of monitoring gene expression quantitatively and qualitatively in a cell using a gene fusion construct coding for a polypeptide having both luciferase and GFP activities, the method comprising the steps of:

- (a) providing a gene fusion construct coding for a polypeptide having both Renilla luciferase and GFP activity;
- (b) introducing the gene fusion construct into the cell;
- (c) maintaining the cell containing the gene fusion construct in a manner allowing the cell to express the polypeptide; and
- (d) measuring the cell for luciferase and fluorescent activity.

20. The method of claim 19, where the step of providing comprises providing a construct including a polynucleotide sequence as set forth in SEQ ID NO:1.

21. A method of monitoring gene expression quantitatively and qualitatively in a cell using a gene fusion construct coding for a polypeptide having both luciferase and GFP activities, the method comprising the steps of:

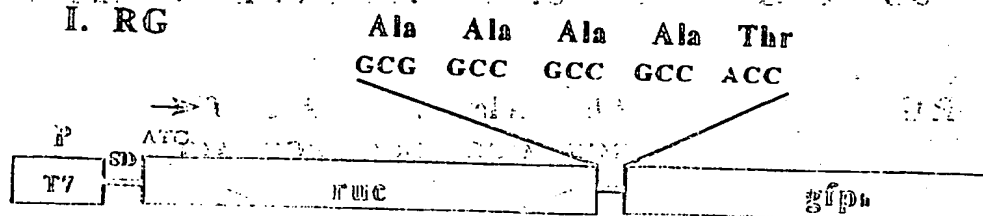
- (a) providing a gene fusion construct comprising the protein of claim 1;
- (b) introducing the gene fusion construct into the cell;

- (c) maintaining the cell containing the gene fusion construct in a manner allowing the cell to express the polypeptide; and
- (d) measuring the cell for luciferase and fluorescent activity.

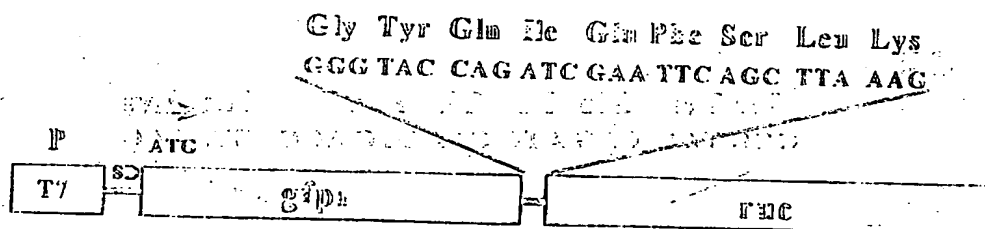
FIG. 1

# Fusion Gene Cassettes for *E. coli*

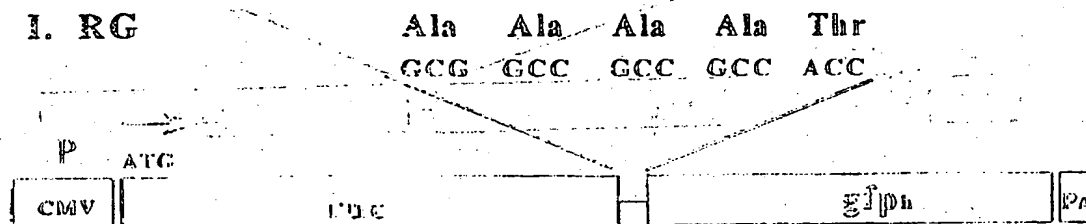
I. RG



II. GR



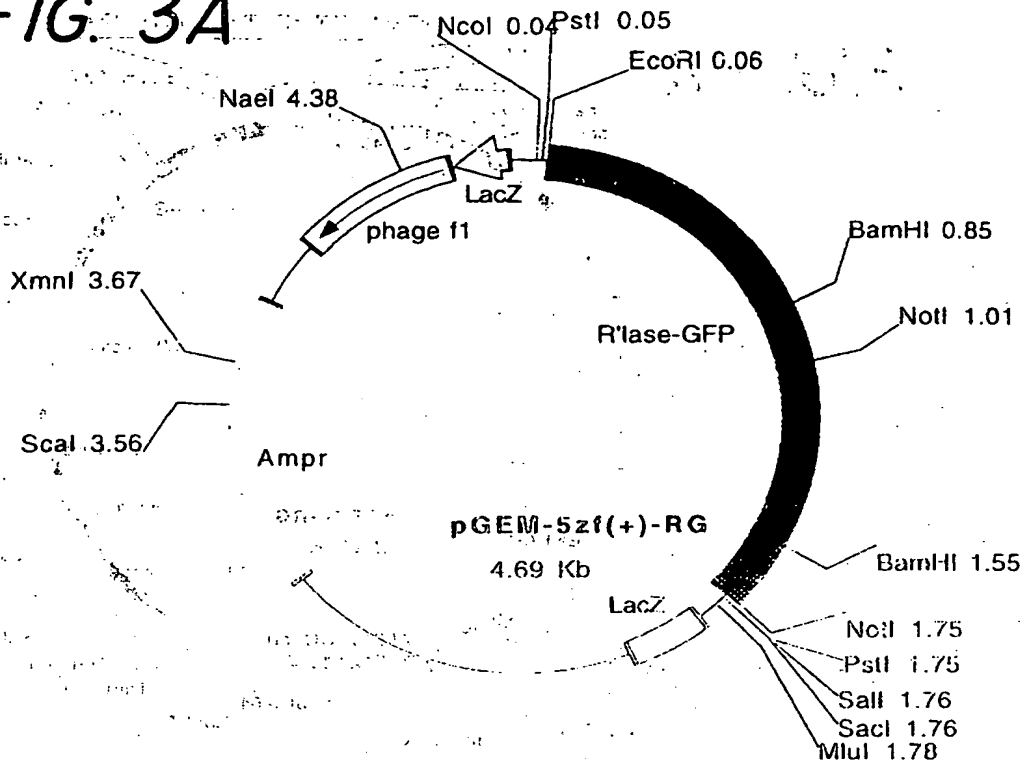


**FIG. 2****Fusion Gene Cassettes for Mammalian cells****I. RG****II. GR**

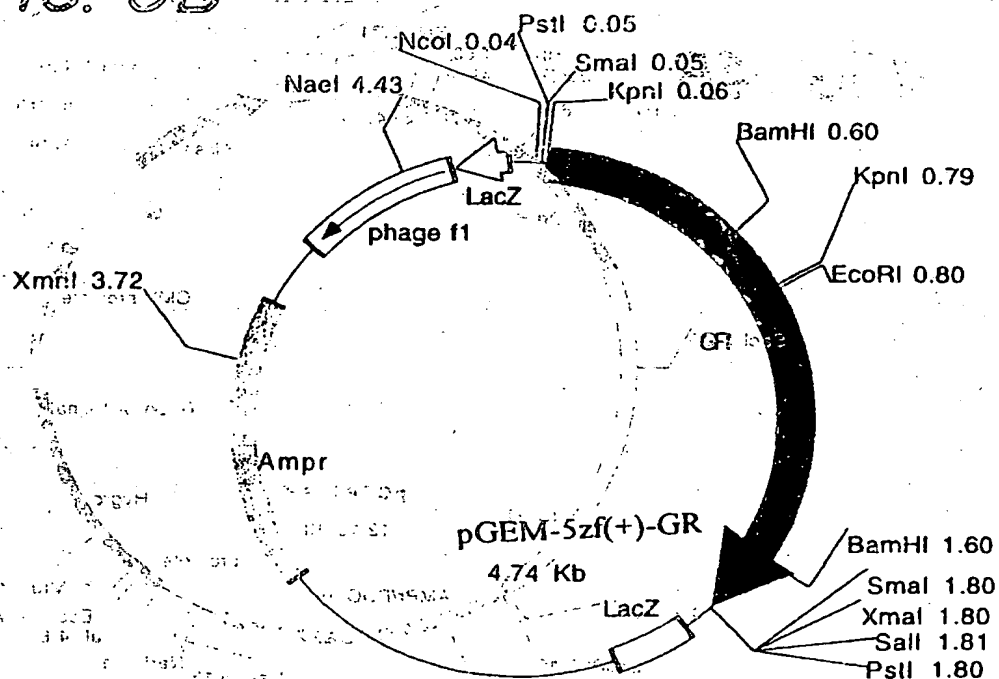
(32 HOURS) 1997 BY BENTLEY &amp; CO.

**SUBSTITUTE SHEET (RULE 26)**

**FIG. 3A**



**FIG. 3B**



SUBSTITUTE SHEET (RULE 26)

FIG. 4A

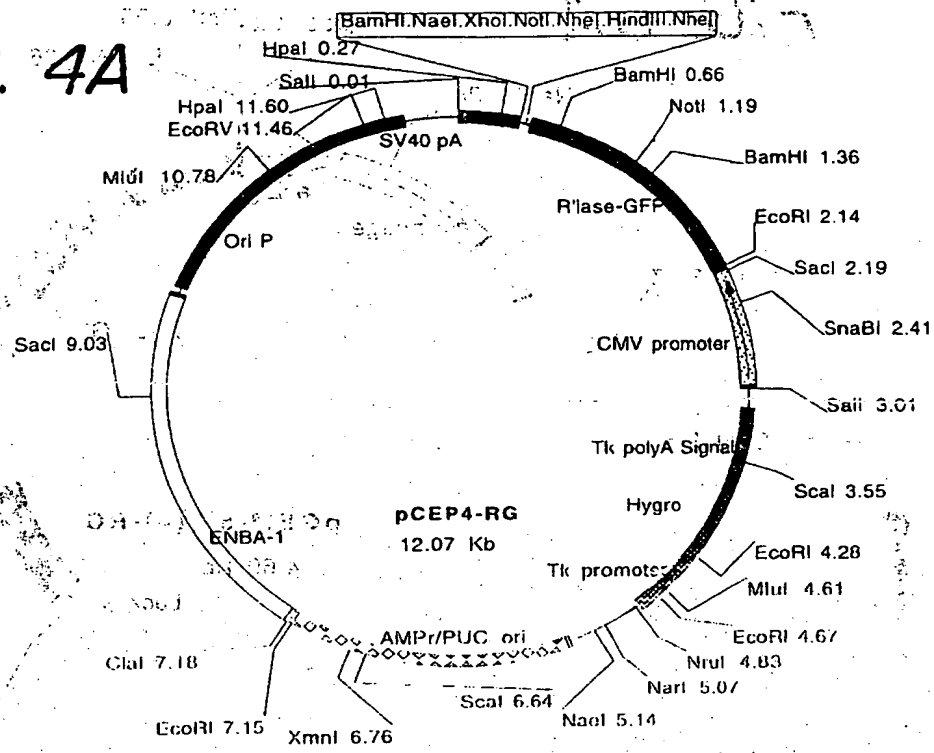


FIG. 4B

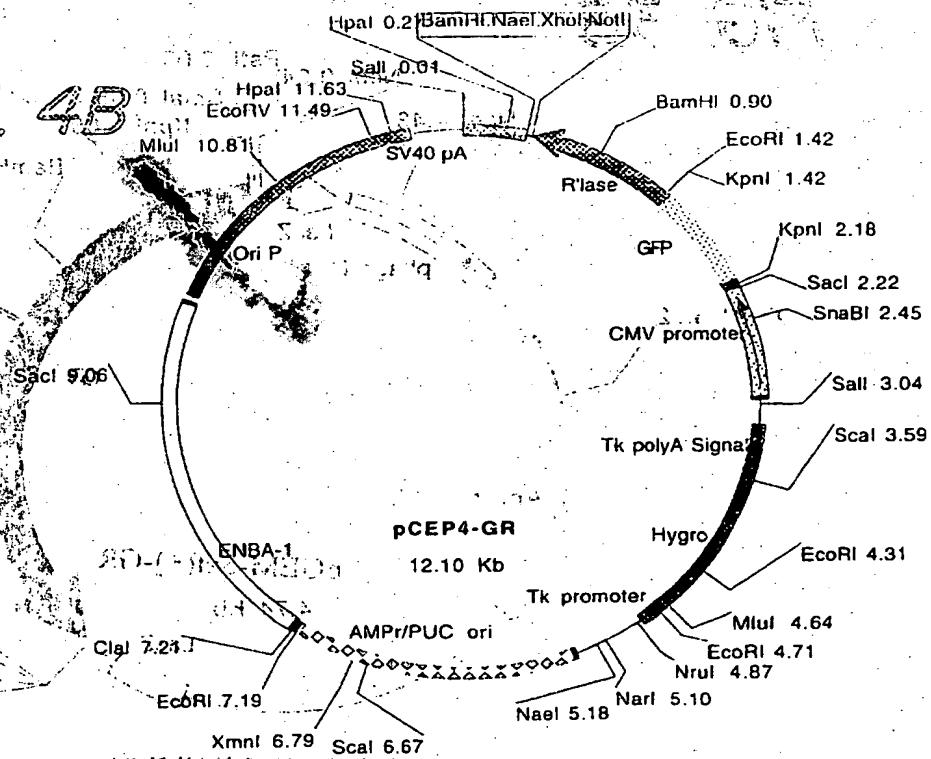


FIG. 5A

FIG. 5A

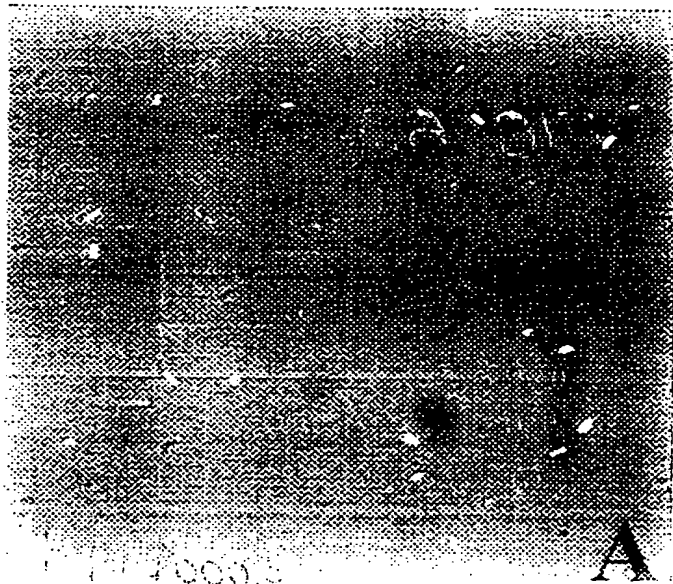
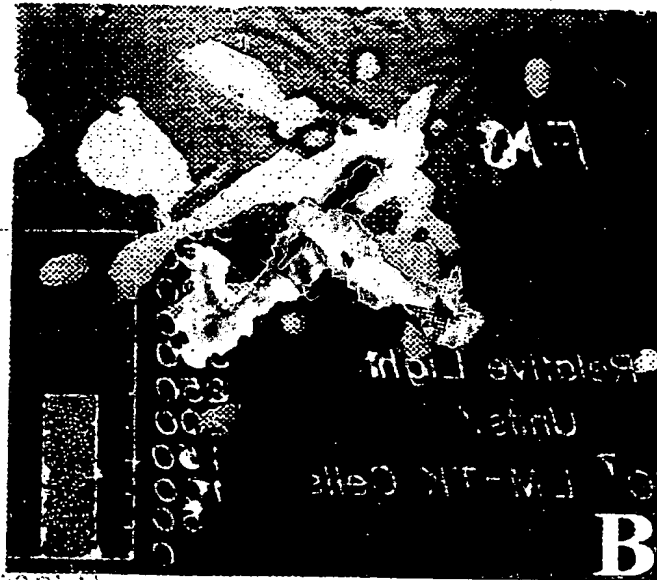


FIG. 5B



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FIG. 6A

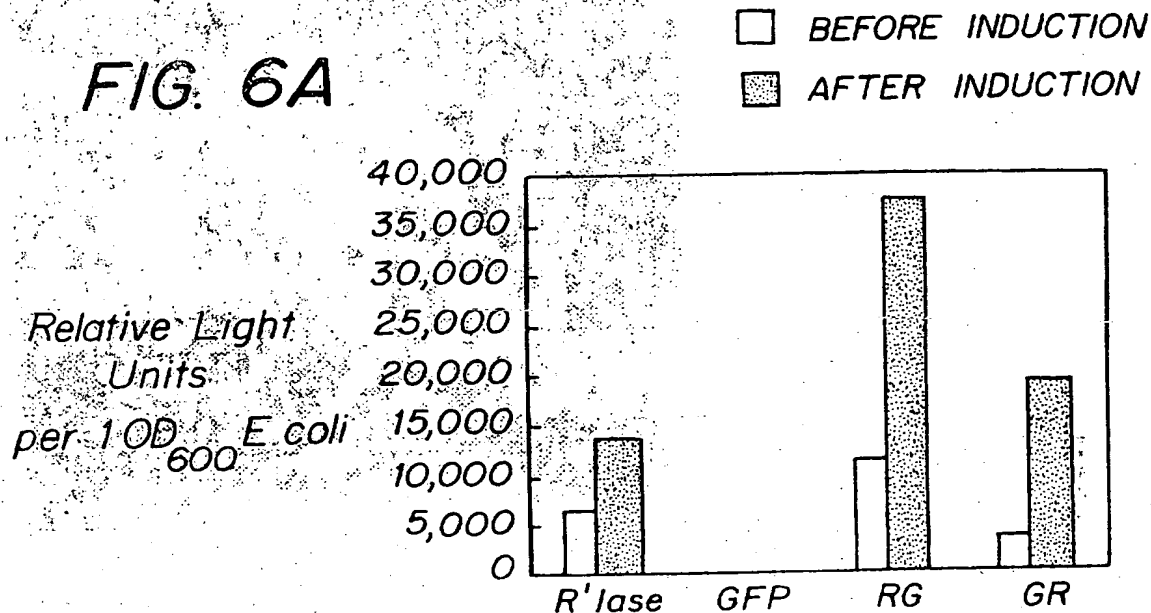


FIG. 6B

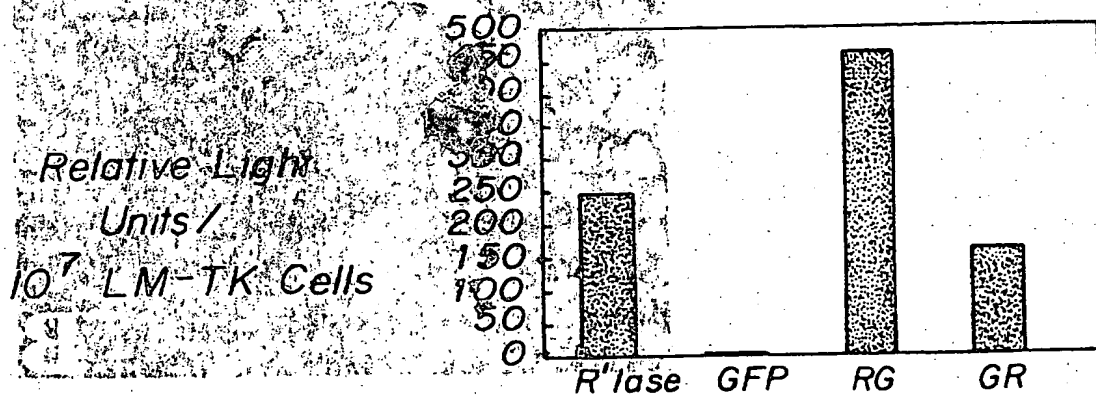


FIG. 7

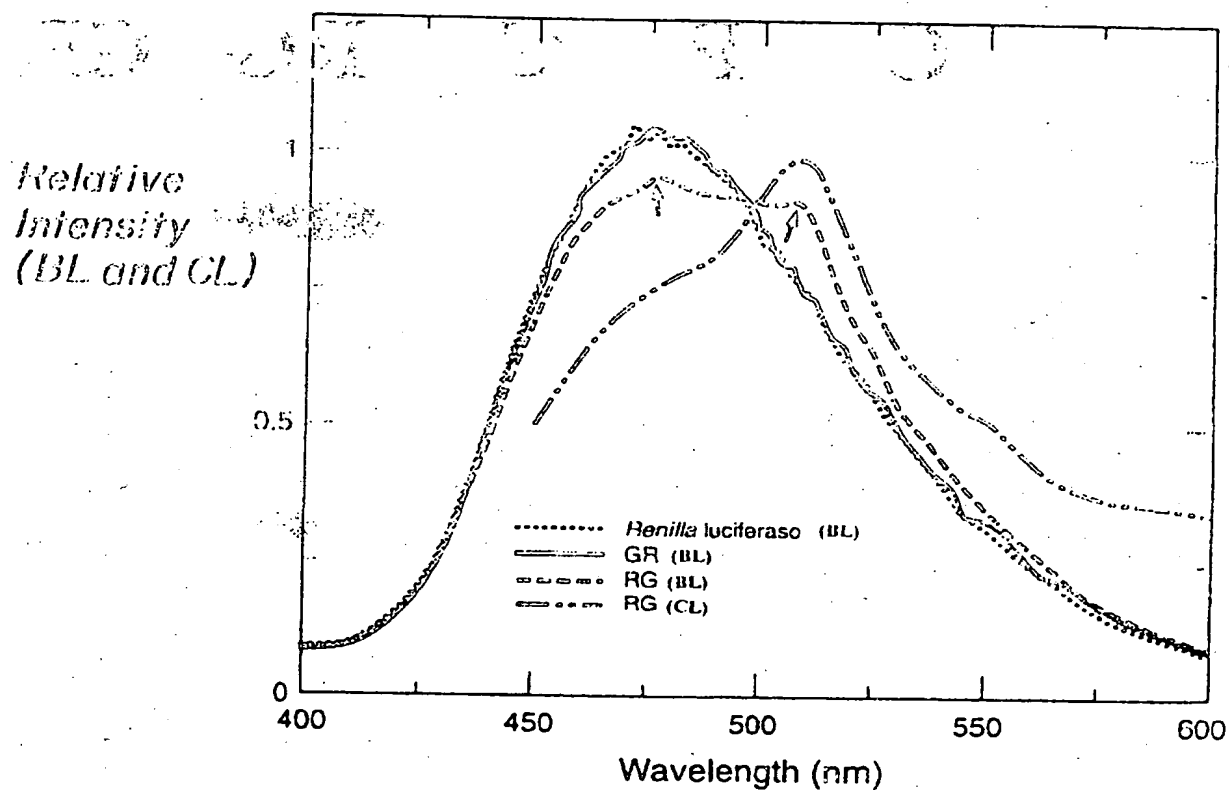


FIG. 9A



FIG. 9B



FIG. 9C



FIG. 9D

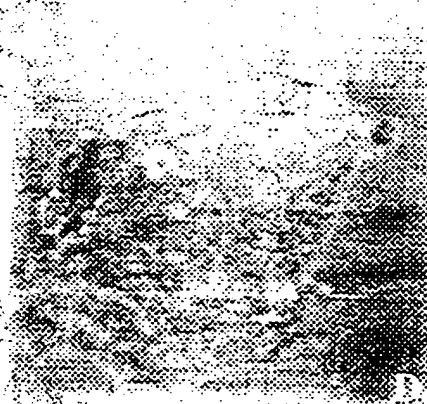


FIG. 9E

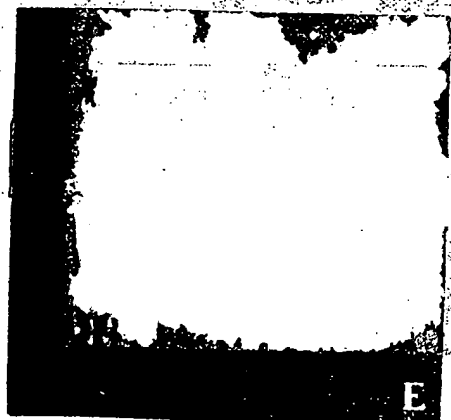


FIG. 9F

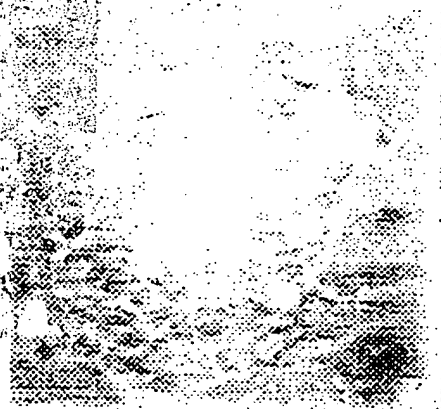


FIG. 8

C R G RG GR

65

34



FIG. 10A

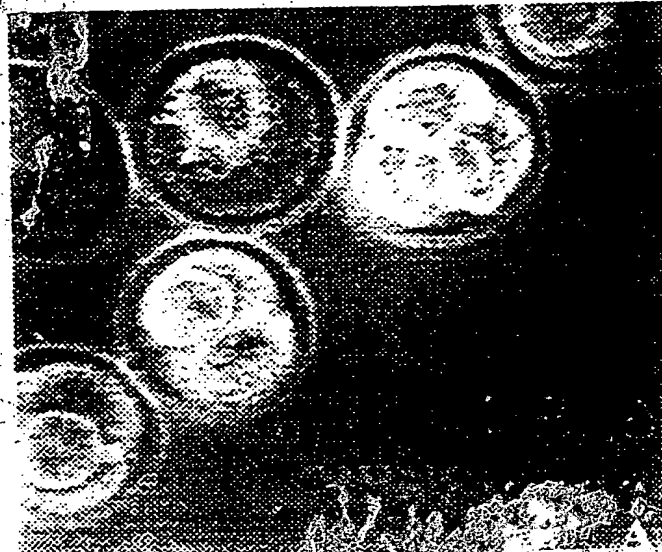
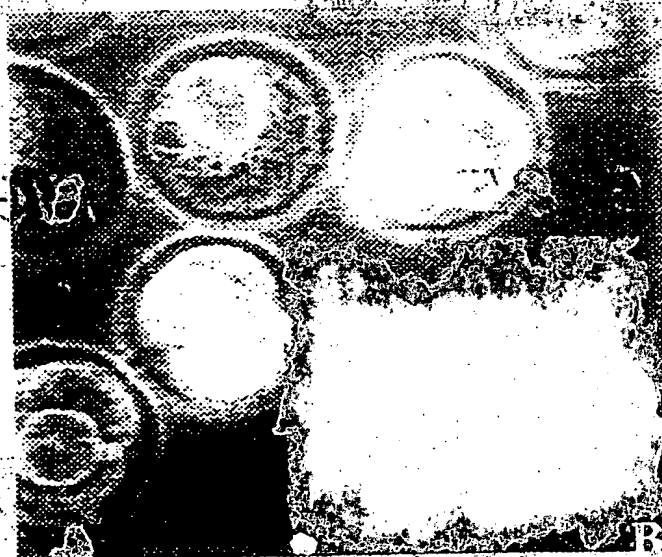


FIG. 10B



(SUBSTITUTE SHEET (RULE 26))

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/17162

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet

US-CL : Please See Extra Sheet

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.7, 189, 252.3, 320.1, 530/350, 388.1, 536/23.2, 23.4, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS(USPAT, EPOABS, JPOABS), STN (CAPLUS, BIOSIS)

search terms: luciferase, green fluorescent protein, renilla, aquorea, DNA, fusion, gene, antibody, monoclonal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y — A	US 5,491,084 (CHALFIE et al) 13 February 1996, entire patent, especially column 1, lines 16-25 and claims	1, 2, 6-9, 11, 13, 15-17, 19-21 3, 12, 14, 20
Y — A	US 5,292,658 (CORMIER et al) 08 MARCH 1994, entire patent, especially claims.	1, 2, 6-9, 11, 13, 15-17, 19-21 3, 12, 14, 20

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

\* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*B\* earlier document published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\*

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\*

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\*

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*A\*

document member of the same patent family

Date of the actual completion of the international search

11 DECEMBER 1997

Date of mailing of the international search report

23 JAN 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. - 20231

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Authorized officer

ELIZABETH FLODY-ANKY

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1992)\*

## International application No.

PCT/US97/17162

## Category:

Citation of document, with indication, where appropriate, of the relevant passages

Relevant to claim No.

**X**

**SANDALOVA, T. Some Notions about Structure of Bacterial Luciferase, Obtained from Analysis of Amino Acid Sequence, and Study of Monoclonal Antibody Binding. In: Biological Luminiscence, Proceedings of International School, 1st (1990), Meeting Date 1989, 330-340. Editors: Jezowska-Trzebiatowska et al. World Science, Singapore, Singapore (Abstract)**

4, 10

**Y.**

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/162

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/17162

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

C12P 21/04, 21/06; C12N 1/20, 9/02, 15/09; C07K 14/00, 16/00; C07H 21/04

## A. CLASSIFICATION OF SUBJECT MATTER: US CL :

435/69.1, 69.7, 189, 252.3, 320.1; 530/350, 388.1; 536/23.2, 23.4, 23.5

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-3, 6 and 7, drawn to a fusion protein having both luciferase and GFP activities.

Group II, claim(s) 4, 5 and 10, drawn to a monoclonal antibody against said fusion protein.

Group III, claim(s) 8, 9 and 11-17, drawn to a DNA encoding said fusion protein, a vector containing said DNA, a cell transformed with the same, a method of producing said fusion protein using a transformed cell and 1st method of use of said DNA.

Group IV, claim 18, drawn to a method of making a monoclonal antibody.

Group V, claim(s) 19-21, drawn to 2nd method of use of DNA encoding fusion protein.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: a fusion protein of Group I, an antibody of Group II and a DNA of Group III are different compounds with different structures, functions and utilities. Luciferase and GFP as well DNAs encoding them and gene fusion constructs based on each of them are known in the prior art. An antibody against both proteins are known. Therefore, a fusion protein containing either luciferase or GFP lacks a special technical feature with a DNA encoding thereof and an antibody against it.

Inventions of Groups IV and V are drawn to materially different methods. Method of Group IV employs immunization of an animal with a fusion protein and a hybridoma production, whereas a method of Group V employs a DNA construct encoding a fusion protein.

PCT Rule 1.475(d) does not provide for multiple products or methods within a single application and therefore, unity of invention is lacking with regard to Groups I-V.

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